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Undifferentiated human mesenchymal stem cells (hMSCs) are highly sensitive to mechanical strain: transcriptionally controlled early osteo-chondrogenic response *in vitro*

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Summary

Objective: Physical cues play a crucial role in skeletogenesis and osteochondral regeneration. Although human mesenchymal stem cells (hMSCs) offer considerable therapeutic potential, little is known about the molecular mechanisms that control their differentiation. We hypothesized that mechanical strain might be an inherent stimulus for chondrogenic and/or osteogenic differentiation in undifferentiated hMSCs, where c-Fos (FOS) might play a major role in mechanotransduction.

Method: hMSCs from 10 donors were intermittently stimulated by cyclic tensile strain (CTS) at 3000 μstrain for a period of 3 days. Differential gene expression of strained and unstrained hMSCs was analysed by real-time RT-PCR for several marker genes, including the transcription factors FOS, RUNX2, SOX9, and others. Additionally, alkaline phosphatase activity (ALP) was determined kinetically.

Results: The application of CTS significantly stimulated the expression levels of the early chondrogenic and osteogenic marker genes (SOX9, LUM, DCN; RUNX2, SPARC, SPP1, ALPL); this was accompanied by stimulation of ALP activity ($+38\% \pm 12$ standard error of mean, $P < 0.05$). Matrix analysis revealed that the osteo-chondrogenic response followed a coordinated expression pattern, in which FOS was attributed to early osteogenic but not chondrogenic differentiation.

Conclusion: Undifferentiated hMSCs are highly sensitive to mechanical strain with a transcriptionally controlled osteo-chondrogenic differentiation response *in vitro*.

Key words: Bone marrow stromal cells, Osteogenesis, Chondrogenesis, Endochondral ossification, c-Fos, Integrin receptors, Mechanical strain, Donor-to-donor variance.

Introduction

Mesenchymal stem cells (MSCs) derived from bone marrow represent an exciting progenitor cell source for applications in cell-based medicine due to their ability to differentiate into several mesenchymal cell lineages, and a number of studies have convincingly shown the feasibility of MSC grafts for tissue engineering purposes[1–3].

MSCs are responsible for the development of the axial and appendicular skeleton in vertebrates. The process of endochondral ossification during skeletogenesis is characterized by a highly coordinated sequence of mesenchymal cell proliferation and condensation, chondrocyte maturation and matrix calcification, and finally the apposition of new bone[4]; this process persists throughout life in regeneration processes (e.g., healing of bone fractures, deep cartilage lesions, in distraction osteogenesis). In acute osteochondral injury, human mesenchymal stem cells (hMSCs) within the bone marrow are thought to migrate into the affected area, where osteogenic and/or chondrogenic differentiation is induced to form new bone and hyaline cartilage-like tissue. Simple surgical methods like drilling through subchondral bone or microfracturing have been successfully introduced to evoke a similar response[5].

The importance of physical factors in the development and maintenance of cartilage and bone tissue has long been recognized. The biological response of tissues to the mechanical environment is the result of a complex mechanotransduction process to accommodate the new loading environment within the limits of normal daily stress stimuli, as hypothesized by Frost *et al.*[6] in bone (re)modelling. Indeed, many authors have shown that bone cells (osteoblasts and osteocytes) are sensitive to physical stimuli[7,8], and that this sensitivity seems to be highly dependent on the differentiation state[9,10]. Similarly, a stimulatory effect was also found in articular chondrocyte monolayer culture[11] as well as in 3D culture systems. Mauck *et al.* demonstrated that dynamic loading of cell–matrix constructs promotes chondrogenesis in addition to the chondrogenic effects evoked by tumour growth factor-β

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1 (TGF-β_1) and IGF-I[12,13]. *In vivo*, increased chondrogenesis was also demonstrated for continuous passive motion[14], while immobilization was shown to be detrimental to cartilage development and repair[15].

Although not yet fully understood, mechanical signal transduction appears to trigger differentiation process through the induction of major tissue-related regulators/transcription factors. Among others, c-Fos (FOS) is a well-known immediate early response gene to mechanical stress in various cell types including osteocytes and osteoblasts[16]. The Fos gene family encodes nuclear proteins that dimerize with Jun family proteins to form the activator protein-1 transcription complex (AP-1), which is engaged in the regulation of major osteoblast-related genes, including those for alkaline phosphatase (ALPL), type I collagen, osteopontin (SPP1), osteonectin (SPARC), and osteocalcin[17]. *In vivo* experiments on bone loading and distraction osteogenesis demonstrated the importance of FOS in the physiological response to mechanical forces[18,19]. Although beyond the primary scope of the present study, it has been suggested that integrin receptors play a major role in mechanotransduction[16,20], too, where the $\alpha v\beta 5$-receptors may be of relevance in mediating strain-dependent chondro-osteogenic response for several reasons: (1) they are known to bind to and act as a receptor for osteopontin[21]; (2) they are capable of mediating chondrocyte adhesion in normal human articular cartilage[22]; and (3) they are highly expressed in the majority of hMSCs[23].

The induction of differentiation is a highly programmed lineage-specific process at the molecular level, and several studies have provided great insight into the microenvironment affecting differentiation of multipotential hMSCs, including hormones, cytokines and growth factors (e.g., TGF-β, insulin, steroids, BMPs) as well as other factors like cell density, scaffold materials, and others[24]. While the role of mechanical environment in chondrocyte and osteoblast phenotype and activity is well established, very few studies have directly investigated the influence of mechanical signals in stem cells, which may be crucial in promoting induction of differentiation pathways (reviewed by Estes *et al.*[25]).

The present study aimed to determine whether mechanical strain inherently provides an early chondro- and/or osteoinductive effect in undifferentiated hMSCs, where FOS may be critically involved at the level of transcriptional regulation.

Materials and methods

EXPERIMENTAL DESIGN

hMSCs were isolated from bone marrow aspirates from five female and five male age-matched donors undergoing elective orthopaedic surgery. The individual transcriptional response of undifferentiated hMSCs to cyclic tensile strain (CTS) was determined in a two-armed study design (strained vs unstrained hMSCs) intended to eliminate possible cofounders not related to mechanical strain. Mechanical stimulation was applied by a computer-assisted four-point bending device, for which uniaxial elongation had been shown to be transferred properly to cell monolayers[26,27]. The magnitude of tensile strain was restricted to a maximum of 3000 μstrain in order to avoid a pathological response due to the application of supra-physiological strains found in monolayer culture systems[28]. The expression pattern of the following set of genes was analysed by real-time RT-PCR: genes associated with osteogenic differentiation (RUNX2, ALPL, SPARC, SPP1), chondrogenic differentiation (SOX9, DCN, LUM, COL2A1, CRTAC1, COL10A1), protein synthesis (COL1A1), cell cycle entry (MKI67)[29], early response genes (FOS, G6PD), and integrin receptors (ITGAV, ITGB5). Additionally, alkaline phosphatase activity (ALP) was quantified kinetically to characterize early osteogenic phenotype.

SUBJECTS

The study was approved by the local Institutional Review Board (*control no. 12-091*) and written informed consent was obtained from all subjects. Patients with a history of myelogeneous disease, a current history of neoplasm, or infections were excluded from the study. Five female and male donors matched for age were enrolled.

MSC ISOLATION AND FACS ANALYSIS

Up to 10 ml heparinized bone marrow aspirates were harvested from the iliac crest at the beginning of elective orthopaedic surgery by a single surgeon. Mononuclear cells' fraction (MNCs) was isolated by gradient centrifugation at 900*g* for 30 min on Percoll (Amersham-Pharmacia, Vienna, Austria; density 1.073 g/ml) as described in detail by Mackay *et al.*[30]. Additionally, FACS analysis revealed the following surface protein expression pattern to be characteristic for the hMSCs used: CD14− (FITC, IgG2a), CD29+ (RPE, IgG1), CD34− (PerCP, IgG1), CD44+ (FITC, IgG2a), CD45− (PerCP, IgG1), CD49e+ (FITC, IgG2b), CD105+ (RPE, IgG1), CD166+ (FITC, IgG1) (data not shown; all antibodies including isotype controls were purchased from Serotec, Vienna, Austria). For cryopreservation, MNCs were resuspended at a concentration of 1×10^6 MNCs per ml in DMEM-LG (Gibco, Vienna, Austria) with 10% dimethylsulfoxide (Sigma–Aldrich, Vienna, Austria) and 2% human albumin. Aliquots of 2 ml were slowly frozen and cryopreserved in liquid nitrogen until use.

CELL CULTURE AND CELL EXPANSION

Cells were cultured at standard conditions of 37°C, 5% CO_2, and 100% humidity in complete growth medium: DMEM/F12 (Gibco, Vienna, Austria) with 10% foetal bovine serum (FBS, lot 40F6120K; Gibco), 2 mM L-glutamine (Sigma–Aldrich, Vienna, Austria), 100 U/ml penicillin/streptomycin (Gibco), and 1 ng/ml amphotericin B (Gibco). Cells were subcultured at 70% confluence until passage 3–5 using Accutase™ (PAA, Pasching, Austria).

Custom-made silastic dishes were preconditioned with complete standard growth medium overnight, washed twice with phosphate buffered saline (PBS) [PAA] and coated with fibronectin (5 μg/ml in PBS, PAA) for 20 min at room temperature (RT). Stability in the gene expression profiles of undifferentiated hMSCs during long-term expansion up to passage 10 was recently shown in large-scale expression profiling[31]. In an effort to avoid contact inhibition and differentiation before the application of CTS, hMSCs were plated at a cell density of 1×10^4 cells per cm^2 after additional washing with PBS. Cells were allowed to attach overnight in complete growth medium supplemented with 0.05 mM L-ascorbic acid 2-phosphate (ASA2P, Sigma–Aldrich) before starting CTS. The same procedures were performed simultaneously without the application of cyclic strain for controls (Co).

CTS

CTS was applied with a computerized custom-made four-point bending device[27] (Cell Strain Unit v3.0, University of

Iowa) using the following strain pattern: 1000 cycles (1 Hz) of sawtooth-shaped uniaxial tensile strain at a magnitude of 3000 μstrain, six times a day for a total period of 72 h. Medium was changed on day 2 and the supernatant carefully examined for non-adherent cells by light microscopy. All measurements started immediately following the last straining cycle.

ANALYSIS OF mRNA EXPRESSION BY REAL-TIME RT-PCR

Total RNA isolation was started immediately after straining, using the Aqua Pure RNA Isolation Kit (Bio-Rad, Vienna, Austria) as recommended by the manufacturer's manual. Total RNA recovery was determined based on UV-spectrophotometric equivalents at A_{260}. Reverse transcription was carried out after treatment with DNAse I (Sigma–Aldrich) using the 1st Strand cDNA Synthesis Kit with random hexamer primers reverse transcription (Roche Diagnostics, Mannheim, Germany). Negative controls were performed without RNA templates and without reverse transcriptase (AMV). Preparations of cDNA were checked by spectrophotometry (A_{260} and A_{280}) and gel electrophoresis. PCR primers were designed using the PRIMER3 software (Steve Rozen, Helen J, Skaletsky 1998 Primer3; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were all chosen to span exon junctions. Specific primer pairs (TIB-Molbiol, Berlin, Germany), PCR product length and calculated melting temperatures are shown in Table I. Real-time PCR was run in a LightCycler Instrument (Roche, Germany) using the FastStart DNA Master SYBR Green I Kit (Roche Diagnostics) with the following program: initial activation of the FastStart Taq polymerase at 95°C for 10 min, followed by up to 35 cycles at 95°C for 10 s, 57°C for 7 s, and 72°C for 7–9 s. PCR efficiency was monitored by an internal cDNA template added to each run. All measurements were performed at least in duplicate, and for each sample the threshold cycle (C_T) values were determined automatically by the LightCycler software (Roche). Specificity of the amplicons was confirmed by a single peak in melting curve analysis within a 1°C limit of calculated melting temperatures (http://www.promega.com/biomath/calc11.htm) in conjunction with agarose gel electrophoresis. The PCR product of clathrin (CLCT) was sequenced using fluorescent automated sequencing (Beckman model CEQ8000, software version 5-0.345). The relative changes in gene expression were determined by the $2^{-\Delta\Delta C_T}$ method as described in detail by Livak *et al.*[32]. Briefly, the ΔC_T value represents the difference between the threshold cycle (C_T) of each target and the housekeeping genes CLCT[33] and β_2-microglobulin (B2MG) ($\Delta C_T = 0$). Comparison of gene expression was derived from subtraction of ΔC_T values of unstrained from the ΔC_T values of strained samples to give a $\Delta\Delta C_T$ value; the CTS-related response in relative gene expression was calculated as $2^{-\Delta\Delta C_T}$, indicating fold change in gene expression relative to the unstrained controls.

ALP ACTIVITY

Standard protocols of conversion of *p*-nitrophenyl phosphate (*p*-NPP) to *p*-nitrophenol (*p*-NP) were used to measure ALP activity (Sigma Kit 104). Cells were lysed with 0.5% TritonX-100 (Sigma–Aldrich) after triple washing with Tyrode's balanced salt solution (Sigma–Aldrich) and lysates were incubated to 360 μM *p*-NPP in 0.75 M ALP

Table I

Real time RT-PCR primer description. RT-PCR was performed as described in the Methods section (MT = estimated melting temperature)

Symbol	Gene	Accession no.	Primer sequence	Amplicon size (bp)	MT (°C)
CLTC	Clathrin, heavy polypeptide (Hc)	X55878	5′-AGAAACTGCATGGAGGCACAA 3′-TGGGGCTGACCATAAACAATG	165	81
B2M	Beta-2-microglobulin	AB021288	5′-GCTATCCAGCGTACTCCAAAGA 3′-GGATGGATGAAACCCAGACA	102	80
COL1A1	Collagen, type I, alpha 1	Z74615	5′-CGAAGACATCCCACCAATCAC 3′-TCCCTTGGGTCCCTCGAC	250	92
RUNX2	Runt-related transcription factor 2	AF001443	5′-ACAGTAGATGGACCTCGGGAAC 3′-TGAGGCGGTCAGAGAACAAA	82	83
ALPL	Alkaline phosphatase, liver/bone/kidney	NM_000478	5′-CCTGGACCTCGTTGACACCT 3′-GTCCCCTGGCTCGAAGAGA	136	86
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	J03040	5′-TGCCACTGAGGGTTCCCA 3′-TCGGTTTCCTCTGCACCATC	211	88
SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	NM_000582	5′-AACGCCGACCAAGGAAAACT 3′-GGCCACAGCATCTGGGTATT	150	84
SOX9	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)	NM_000346	5′-AGTACCCGCACTTGCACAAC 3′-CCGTTCTTCACCGACTTCCT	178	93
DCN	Decorin	NM_001920	5′-CAATGCCATCTTCGAGTGGT 3′-GCGTGAAGGTTCTTCAGGTTC	152	84
LUM	Lumican	NM_002345	5′-CCGTCCTGACAGAGTTCACAG 3′-TGGCAAATGGTTTGAATCCT	111	79
FOS	c-Fos	XM085152	5′-GCTTTGCAGACCGAGATTGC 3′-TTGAGGAGAGGCAGGGTGAA	203	90
G6PD	Glucose-6-phosphate dehydrogenase	NM_000402	5′-TGCCTTCCATCAGTCGGATA 3′-GCCTTGAAGAAGGGCTCACT	198	89
MKI67	Antigen identified by monoclonal antibody Ki-67	X65550	5′-GCCTGTACGGCTAAAACATGGA 3′-TTGAGGAGAGGCAGGGTGAA	182	82
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	NM_002210	5′-TGGGTTATTCTGTGGCTGTCG 3′-AGCCATCTGCTCGCCAGTAA	155	83
ITGB5	Integrin, beta 5	NM_002213	5′-TTGGCAGAGAACAACATCAACC 3′-TCCTCAGGCTGATCCCAGAC	200	82

buffer (pH 10.3; Sigma–Aldrich) at 37°C. Quantification of *p*-NP was carried out kinetically at 405 nm within the linear range using a *p*-NP standard absorption curve. Diluted FBS was used for positive control. Cell numbers were determined by two independent investigators via haemocytometer after viability stain with trypan blue and mean values were used for normalizing.

DATA ANALYSIS

Deviations from normal distribution were tested with the Kolmogorov–Smirnov test. Statistical analysis of ALP activity was performed by the paired samples *t* test, differential gene expression was analysed by the Wilcoxon signed-ranks test, and the correlation matrix was calculated by Spearman rank correlations. Values are presented as mean ± standard error of the mean (S.E.M.) or as median value with 95% confidence interval (CI). Results were considered significant at $P < 0.05$.

Results

BASELINE CHARACTERISTICS

hMSCs of 10 donors designated for elective orthopaedic surgery were selected for the study. Ages ranged from 43 to 85 years (mean 67.9 ± 4.9 years) without significant differences between female and male donors (66.1 ± 6.8 years and 69.7 ± 7.9 years, respectively).

CHARACTERIZATION OF UNSTRAINED hMSCs (CO)

ALP activity ranged between 0.89 and 11.18 μM/min/10^6 cells (mean 6.14 ± 1.51 μM/min/10^6) without significant differences between female and male donors (5.5 ± 2.5 μM/min/10^6 and 6.5 ± 2.1 μM/min/10^6, respectively).

Donor-to-donor variance was reflected by up to 218-fold differences in mRNA levels of ALPL (Fig. 1). The highest gene expression levels were found for SPARC, whose median value was approximately 800-fold higher than the median level found for the transcription factor SOX9. No mRNA expression was detected for the late chondrogenic markers COL2A1, COL10A1 and CRTAC1. Again, we did not find any significant differences between gender or relations to donor ages.

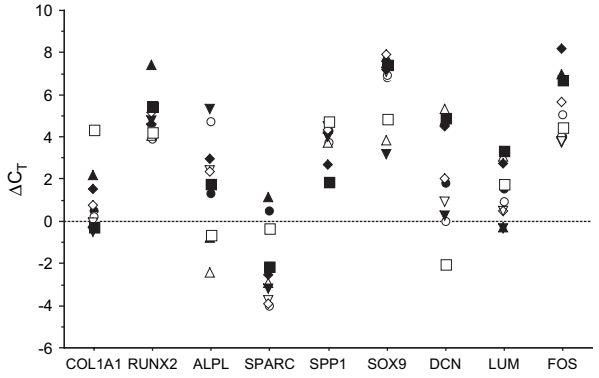

Fig. 1. Relative gene expression levels of unstrained hMSCs (Co). Each symbol represents one of 10 sex- and age-matched donors. The figure demonstrates transcriptional heterogeneity and pronounced donor-to-donor variance in hMSCs.

EFFECTS OF CTS

ALP activity was significantly stimulated by CTS: +38.4 ± 12% ($P < 0.05$), ranging from −6% to +94% in individual samples.

The response on mRNA expression levels to CTS showed marked inter-individual differences (Fig. 2); e.g., the mRNA levels of ALPL varied between −33% and +767% of values determined in unstrained hMSCs (median +165%, $P < 0.05$). As indicated in Table II, a significant stimulatory effect was also found for COL1A1 (3.5-fold), for the osteogenic markers RUNX2 (1.9-fold), ALPL (2.5-fold), SPARC (3.8-fold), SPP1 (2.6-fold), and the chondrogenic markers SOX9 (3.1-fold), DCN (2.6-fold), LUM (2.3-fold). Messages of G6PD (2.6-fold), ITGAV (2.1-fold), and ITGB5 (2.4-fold) were significantly up-regulated as well, but failed statistical significance in FOS (1.6-fold; P 0.106; range from −36% to +517%). No stimulatory effect was found for MKI67 (0.6-fold), and COL2A1, COL10A1 and CRTAC1 remained undetectable following CTS.

ANALYSIS OF GENE EXPRESSION PATTERN

The calculation of the correlation matrix was performed on relative gene expression levels (ΔC_T) found within (1) unstrained (Co) and (2) strained hMSCs (CTS), as well as (3) the magnitude of CTS-related changes ($-\Delta\Delta C_T$). All significant correlations ($P < 0.05$) found within unstrained and strained hMSCs are given in Table IIIA: In Co, positive correlations were found between SOX9 and FOS ($r = 0.73$)/RUNX2 ($r = 0.69$) messages, while RUNX2 expression levels were highly correlated to the FOS messages ($r = 0.96$) within CTS. CTS-related dependences in gene expression pattern are summarized in Table IIIB: RUNX2 messages of Co were positively correlated to FOS expression levels in CTS ($r = 0.75$). Additionally, the mRNA levels of SPP1 determined in Co were predictive for the mechano-stimulatory effect on FOS ($r = 0.67$), and the magnitudes of CTS-related stimulation of both genes were positively correlated to each other ($r = 0.82$). Similarly, SOX9 messages of Co were positively correlated to DCN expression ($r = 0.67$) following CTS, while the messages of DCN and LUM in Co were inversely related to the magnitude of the individual CTS-related stimulation of SPARC ($r = -0.76$) and SPP1/FOS ($r = -0.76/-0.86$), respectively. Additionally, several significant correlations were also found between ITGAV/ITGB5 and the chondrogenic/osteogenic marker genes.

Discussion

We hypothesized that mechanical strain *per se* may be a major stimulus for the induction of chondrogenic and/or

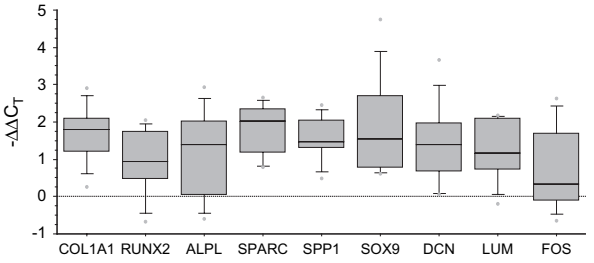

Fig. 2. Response of hMSCs to CTS. All mRNA expression levels of the chondrogenic and osteogenic marker genes were significantly up-regulated in the response to CTS ($P < 0.05$), with the exception of FOS ($P = 0.105$).

Table II
Baseline characteristics and the stimulatory effects of cyclic tension strain. Relative quantification of gene expression levels in unstrained hMSCs (Co) are expressed as ΔC_T values as described in the Methods section. Differential gene expression in the response to CTS was tested by Wilcoxon signed-rank test

Gene	ΔC_T (Co)	95% CI	$-\Delta\Delta C_T$	95% CI	Two-tailed *P*
G6PD	3.4	1.3 to 5.9	1.32	0.97 to 1.81	0.0020
MKI67	6.2	5.4 to 7.4	−0.74	−2.23 to 1.75	NS
COL1A1	0.4	−0.3 to 2.2	1.83	1.15 to 2.26	0.0020
RUNX2	4.5	4.0 to 5.4	0.87	0.23 to 1.55	0.0195
ALPL	2.0	−0.8 to 4.7	1.33	0.31 to 2.02	0.0195
SPARC	−2.8	−4.0 to 0.5	1.89	1.25 to 2.39	0.0020
SPP1	4.1	2.6 to 4.4	1.53	1.10 to 1.99	0.0020
SOX9	6.9	3.8 to 7.8	1.65	0.88 to 2.86	0.0020
DCN	1.9	0.0 to 4.9	1.39	0.70 to 2.18	0.0020
LUM	1.2	−0.4 to 3.0	1.23	0.59 to 2.02	0.0039
FOS	4.8	3.7 to 6.9	0.66	−0.10 to 1.51	NS
ITGAV	2.1	1.0 to 7.1	1.03	0.05 to 1.83	0.0469
ITGB5	4.4	2.9 to 6.0	1.35	0.94 to 1.93	0.0020

Table III
Spearman rank correlations. The correlation matrix was calculated based on gene expression levels (ΔC_T) found (A) within (1) unstrained (Co) and (2) strained hMSCs (CTS), and (B) (3) the magnitude of CTS-related changes ($-\Delta\Delta C_T$). All correlations found with a P-value < 0.05 are listed in order to Spearman correlation coefficient (r)

A. Correlations within unstrained (Co) and strained (CTS) hMSCs

			r	*P*
ΔC_T (Co)				
COL1A1	—	ITGAV	0.86	0.014
FOS	—	SOX9	0.73	0.016
RUNX2	—	SOX9	0.69	0.044
SPARC	—	ITGB5	0.66	0.038
ΔC_T (CTS)				
FOS	—	RUNX2	0.96	<0.00001
SOX9	—	ITGAV	0.89	0.007
COL1A1	—	ITGB5	0.79	0.006
SPARC	—	ITGB5	0.66	0.038
COL1A1	—	ALPL	−0.66	0.038
SPP1	—	LUM	−0.65	0.043

B. CTS-related correlations between strained (CTS) and unstrained (Co) hMSCs

			r	*P*
ΔC_T (Co)		ΔC_T (CTS)		
SPARC	—	ITGB5	0.88	<0.001
SPARC	—	COL1A1	0.77	0.009
RUNX2	—	FOS	0.75	0.013
ITGAV	—	SPARC	0.75	0.052
ITGAV	—	SPP1	0.75	0.052
ITGB5	—	DCN	0.72	0.019
ALPL	—	COL1A1	−0.72	0.019
SPP1	—	LUM	−0.71	0.022
ITGAV	—	COL1A1	0.71	0.071
LUM	—	SPP1	−0.69	0.029
SOX9	—	DCN	0.67	0.033
ΔC_T (Co)		$-\Delta\Delta C_T$		
LUM	—	FOS	0.86	0.002
FOS	—	ITGB5	0.77	0.009
DCN	—	SPARC	0.76	0.011
LUM	—	SPP1	0.76	0.011
ALPL	—	COL1A1	0.75	0.013
SPP1	—	FOS	−0.67	0.033
SPARC	—	SPARC	0.67	0.033
DCN	—	ITGB5	0.64	0.048
$-\Delta\Delta C_T$		$-\Delta\Delta C_T$		
ALPL	—	LUM	−0.89	<0.001
SOX9	—	ITGAV	0.86	0.014
FOS	—	SPP1	0.82	0.004
SOX9	—	DCN	0.71	0.043

osteogenic differentiation in undifferentiated bone marrow derived hMSCs. We found a significant upregulation of G6PD expression due to the application of CTS (+156%, $P < 0.05$), which is in accordance with previous findings of an almost immediate strain-related increase[34], thus indicating that mechanotransduction is brought about successfully in our *in vitro* system. We also found a significant CTS-related stimulatory effect on the major transcription factors known to be critically involved in chondrocyte and osteoblast differentiation, i.e., SOX9[35] (3.1-fold) and RUNX2[36] (1.9-fold), respectively. This was accompanied by a significant increase in the mRNAs encoding for the small interstitial proteoglycans LUM and DCN, both known to be rapidly up-regulated in early chondrogenic differentiation of hMSCs *in vitro*[37], as well as in COL1A1, ALPL, SPP1, and SPARC, all of which are well-known markers associated with early osteoblast differentiation (Fig. 2). Additionally, the osteogenic effect of CTS was confirmed by a concomitant increase in ALP activity (+38%, $P < 0.05$). These results convincingly demonstrate that physical strain represents a strong transcriptional stimulus for both an early chondrocyte and osteoblast differentiation in undifferentiated hMSCs.

However, marked donor-to-donor variability was found in the baseline characteristics of undifferentiated hMSCs (Fig. 1) as well as in the response to CTS (Fig. 2). In general, one major limitation in studying multipotential hMSCs is brought about by their well known and pronounced intrinsic heterogeneity, which is thought to represent lineage hierarchy, where some of the cells are multipotential stem cells while others are more restricted primitive progenitor cells of several cell lineages at various differentiation states[38–40]. Differentiation processes in hMSCs were found to be not synchronized throughout the cell populations[41], and several pheno- and genotypic investigations suggested that early differentiation may be regulated, at least *in vitro*, by stochastic mechanisms, while gene expression programs underlying late events in cell differentiation appear to be more fixed[42,43]. This is thought to represent the high developmental flexibility found in undifferentiated stem cells, which is lost during the commitment to a more mature state in response to strong instructive signals, resulting finally in a specific cell phenotype.

To elucidate whether the chondro-osteogenic differentiation response followed rather a stochastic or cell-line specific pattern, a matrix analysis was performed. We did not find any cell-line specific gene expression pattern (Table IIIA) in unstrained hMSCs, which is in accordance with the characteristic intrinsic heterogeneity of undifferentiated hMSCs. However, previous findings confirmed that even single cell derived hMSCs express markers of multiple cell lineages[44], which may also be responsible for the co-expressions found between SOX9 and RUNX2/FOS, thus reflecting rather differences in the abundance of multipotential hMSCs between particular donors. In contrast, a highly significant co-expression was found between RUNX2 and FOS ($r = 0.96$, $P < 0.00001$) in strained hMSCs. Together with the co-stimulatory effect of CTS in FOS and SPP1 expression ($r = 0.82$, $P = 0.004$), these findings most likely

imply the involvement of FOS in mediating the osteogenic response in hMSCs. Furthermore, the CTS-related response on FOS expression levels seems to be dependent on donor-specific characteristics in SPP1 and LUM expression ($r = +0.67$ and $r = -0.86$, respectively), whereas no dependences were found between FOS messages of unstrained stem cells and either the chondrogenic or the osteogenic response. These findings further support the suggestion that stochastic mechanisms may trigger the expression pattern in unstrained hMSCs due to the lack of inductive differentiation stimuli, while the osteogenic response was attributed to the response in FOS expression, even though not up-regulated significantly ($P = 0.106$) due to CTS. This is in broad accordance with previous findings showing that FOS plays a major role in signalling mechanostimulation in bone cells[16,19,45]. However, very little is known about the role of FOS in chondrogenic differentiation.

Recently, it has been demonstrated that the application of mechanical load augments the expression levels of RUNX2 in hypertrophic chondrocytes or articular cartilage, which was accompanied by an increase in expression levels of the AP-1 partners pc-Jun and c-Fos[46]. In any case, RUNX2 and FOS are known to play a crucial role in late chondrocyte maturation, and we were not able to find any significant relations of FOS to the chondrogenic response in the present study (Table III), but the osteogenic response was inversely dependent on donor-related characteristics of the chondrogenic markers LUM (- SPP1 and FOS) and DCN (- SPARC). However, no such dependences were found for the chondrogenic differentiation effect of CTS. As a result, this suggests that mechanical strain may stimulate early chondrogenic differentiation more directly, while the osteogenic response seems to be under the suppressive control attributed to chondrogenesis, and thus resembles physiological osteo-chondrogenic regeneration *in vivo*, where osteoblasts never appear before chondrocyte maturation has progressed[4]. This may also be in accordance with findings in mouse knee joints, where the accumulation of DCN in uncalcified cartilage with advancing age was suggested to inhibit matrix mineralization, and consequently osteogenesis, at the osteochondral junction side[47].

In contrast to our findings, Huang *et al.*[48] suggested that the activity of AP-1 may be involved in the early stage of MSC chondrogenesis, too. In cell-agarose constructs, they found a transiently weak expression of FOS following 1 h of cyclic compression loading only, while no expression was detected in samples subjected to 2- and 4-h loading, where chondrogenic markers like collagen type II achieved highest levels. Although comparability to our study is limited by significant differences in the study design, based on the aforementioned results it may be speculated that the inherent chondroinductive effect of 3D agarose constructs[49] may have reduced osteogenic capabilities of MSCs necessary for a response in FOS expression, which may have been additionally suppressed beyond the detection limit by promoting chondrogenic differentiation in the longer loaded samples. However, mechanotransduction mechanisms of hMSCs remain obscure and more work is needed to further elucidate the involvement of early response elements like FOS in the differential response of stem cells.

In an effort to avoid biasing CTS-dependent effects by factors influencing early differentiation of hMSCs, all experiments were performed under standard growth culture conditions that were identical for strained and unstrained hMSCs. While the power to detect differences may be significantly decreased due to spontaneous (not CTS-related) induction of osteo-chondrogenic differentiation throughout the experimental procedure, the pronounced strain-related changes found seem to be sufficient to support reliable results in our *in vitro* system. Even though the reasons for donor-related heterogeneity in the molecular characteristics of hMSCs are still unknown, recent findings suggest that this may also reflect considerable alterations in their functional properties *in vivo*[50,51]. Hence, in conjunction with our findings it may be speculated that the simplicity of a reliable *in vitro* model may provide valuable information concerning regeneration capabilities, and it accentuates the need for more functional definition of the hMSCs.

In summary, based on the characteristic donor-related heterogeneity of undifferentiated hMSCs, the application of mechanical strain promoted early chondrogenic and osteogenic differentiation *in vitro*. This osteo-chondrogenic effect of CTS was found to follow a highly controlled transcription pattern, where FOS was attributed to the osteogenic but not the chondrogenic response.

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